Determining Morphological Traits and Genetic Diversity of Rose Aphids Using RAPD and RFLP-PCR Molecular Markers

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Abstract

Rose is one of the most beautiful attractive flowers of world which is important in landscaping because of its unique botanical specifics. One of the most important pests of this plant is aphid. In this study, 135 aphid samples were collected from various regions of Isfahan landscapes. Morphological traits and mitochondrial gene sequencing were used for identifying them. According to morphological traits, these samples were belonging to *Macrosiphum rosae,aphis gossipy,* and *metopolophium dirhodum* species which diversity in their morphological traits was obvious. Studying the genetic diversity of 16 selected samples was done by RAPD-PCR molecular marker using three primers and RFLP-PCR using restriction enzyme *Rsa*I. Results showed high genetic diversity in studying population. Samples grouping were done better by RFLP marker than RAPD marker. So, all samples which were located in three groups by this method had also high relations in morphological traits. On the other hand, genetic differences were shown better by RAPD for insects of a group which didn't have similar morphological traits. Then, this method can be used to observe the highest diversity level of population.

Keywords: Rose , RAPD , RFLP-PCR

1. Introduction

Aphids, as an important group of insects which are belonged to Hemiptera, are very successful creatures with the most species diversity in temperate regions and worldwide distribution. There is few plant species in this area without any specific aphid [1] . They may cause loss of plants directly or indirectly. Plus the direct loss which is made by heavy feeding from sap and includes weakness of plant and finally reduction in yield, they cause indirect loss by honeydew secreting on leaves and branches which absorb dusts and also mold will start to grow and finally photosynthesis and yield will be reduced. Furthermore, aphids are very important economically because of transferring plant viruses and their related diseases [2 , 3] There have been identified more than 4000 insects and this number is increasing daily. Aphids are a little group in proportion to other insects groups but their diversity is very high because of polymorphism existence and creating new biological types [1]. Classification of aphids is according to their morphological traits like other insects. It means that main differences or main similarities of samples are being

compared and they are being located in their own location [2] . Phylogenic classification is based on evaluation history of species, genera, and families and classifies them according to common ancestors and hosts. In this type of classification, apomorphic traits are used [4]. Primary classifications were not based on phylogenic specifics and were according to personal tastes because the importance of phylogenic specifics was not known. Aphid classification is being discussed very much today especially in one main category: their family numbers[4] . .There have been many morphological anatomical studies conducted on aphids which have prepared background for systematic studies about them [5] . Aphids have high ability for adaption and changing and their morphology is being affected by environmental factors. Many ecological physiological factors affect morphological form of aphids [6] . Considering rich vegetation coverage in Iran and high diversity in roses, many aphids' species are not collected yet and probably this diversity in vegetation coverage and weather conditions have been led to high genetic diversity in aphid population of Iran. Molecular methods are appropriate way for responding main basic questions about genetic diversity and more systematic relationships about live organisms. because of high inter species and intra species diversity in populations of some insects like tripses, white flies, and aphids, morphological tool is not efficient for dividing them, then using molecular markers can be very effective [7, 8] . In high taxonomic levels, to study phylogenic relations and also creating and determining classification systems, molecular markers are efficient resources. In molecular level, they help highly in expanding the concept of species. In population level, they explain direct relationship between heredity patterns, distribution, and colonization with temporal geographical distribution. Molecular markers have been used in last years to basic and applied studies of various organisms. So that discovering various molecular markers have caused high progresses in genetic studies [9] . DNA molecule is the base of genetic differences between two live organism and DNA fingerprint is one of the current methods for determining biological identity of live organisms. By comparing electrophoresis profile of DNA we can realize their differences. DNA polymorphism is the base of many genetic studies [10] . Some of PCR based DNA markers are: RAPD, PCR-RFLP, and AFLP [11] . This markers have been used for various purposes like: creating genetic maps, map of traits in diversing populations, saturating genome places with marker , people fingerprints, germplasm analysis, measuring the genetic distance of people, and evaluating parents portions in back cross. This method is a valuable tool in molecular genetics science which is used easily in map creating and fingerprint application [12] . There has not reported any research about morphological and genetic differences of colorful biotypes of rose aphids and then many researches can be done in this category. These studies can be a good introduction for next studies about resistance of rose varieties to these biotypes, their biological differences and their geographical distribution [13] . These types of researches cause more knowledge about pests and then we can find better ways for fighting against pests.

2. Materials and Methods

2.1. Sampling and counting aphids

In order to determine the number of rose aphids, sampling was done from Isfahan province. Because that aphid colonies take place mostly in 10 -15 cm end of branches, about 15 cm of twigs were cut and locate in plastic bags. Sampling was done from young branches because aphids

are interested mainly to this type of branches. Samples were transferred to laboratory and their insects were swept by brush and were stored in 70% alcohol.

2.2. Morphological characterization of aphids

At first, microscopic slides were prepared from healthy samples using boiling with Canadabalsam method. Samples identification was done using different identification keys extant for Iran aphid fauna.

2.3. DNA extraction of roses

DNA extraction was conducted using Kawasaki method (2005) with a little change: aphids were washed by distilled water and located in 1.5 ml micro tubes plus 100 micro liter extraction buffer. Then samples were grinded for one minute to obtain a homogenized suspension. After that another 200 micro liter extraction buffer and 30 micro liter of protease K were added to each tube and were incubated at 60°C for one hour. Then 300 micro liter phenol - chloroform was added to each micro tube and tubes were centrifuged for five minutes in 1200g. Upper part was collected, was transferred to new micro tube with 300 micro liter of phenol – chloroform and then centrifuged again. Then, upper part was mixed with 300 micro liter of Isopropanol and 30 micro liter of sodium – acetate (3M) and was incubated for ten minutes at -20°C. Then samples were centrifuged for ten minutes at 4°C in 2000g. Finally 300 micro liter of 70% ethylic alcohol was added to depsit and were centrifuged in 2000g for ten minutes at 4°C. After eliminating alcohol, micro tubes were dried and 25 micro liter of twice sterilized distilled water was added to them and were kept at -20°C.

2.4. RAPD-PCR reaction for evaluating genetic diversity of aphids

Polymerase chain reaction was conducted using prepared mixture made by Amplicon Company (Japan). Substances of reaction were Perimx: 12.5 micro liters, primer: 0.5 ml, 7 micro liters of twice distilled water, pattern DNA: 1 micro liter. Three primers were used in this study: UBC90 (5 -GGGGGTTAGG), R108 (5 -GTATTGCCCT), and R157 (5 -GCTGTAGTGT). Thermal plan of RAPD reaction was: six minutes at95°c, 40 cycles with 45 seconds at 94°c, 90 seconds at 32°c, 90 seconds at 72°c, and final amplification with 8 minutes at 72°c.

Evaluating of PCR product was done on Agarose gel (1.2%). Nine micro liters of each reactions product plus one micro litter loading buffer (6X from 0.25%Boromo phenol blue and 40% w/v of sucrose) were poured in gel wells and electrophoresis was done at 2.5 volts/cm. After electrophoresis, gel was colored in methyl bromide solution (1µg/ml) for 10-15 minutes and then was decolorized in distilled water for 5-7 minutes. RAPD bands were observed under UV lamps and were shot using Uvtech machine. Evaluating of PCR product was done on Agarose gel (1.2%). Execution buffer of electrophoresis machine was TAE (0.04M Tris-acetate and 0.001 M Na_2EDTA).

2.5. Data analysis of RAPD-PCR

To data analysis of electrophoresis results, existence or none existence of each band was recorded in Excel as one and zero, respectively. Cluster analysis of isolates was conducted using UPGMA method and *NTSYS V 2.2* program.

2.6. RFLP-PCR reaction

One of the molecular markers which are used for diversity studying is pattern of amplified genome segments or RFLP. To this, lepF: 5 -ATTCAACCAATCATAAAGATATGGG-3 and lepR: 5 - TAAACTTCTGGATGTCCAAAAAATCA-3 primer pairs were used in polymerase chain reaction.

PCR substances were: PCR buffer, dNTP (200MM), $Mgcl_2$ (2MM), primers (25 pichomole of each), DNA polymerase Taq (1.25) and pattern DNA (100 nanog.). Primary denaturing of PCR was done for 6 minutes at 95°_{C}, amplification in 35 cycles as 0.75 minute at $94^{\circ}_{C,}$ 1.5 minutes at $55^{\circ}_{C,}$ 1.5 minutes at 72°_{C} and final amplification 5 minutes at 72°_{C}.then,amplified segments were cut in enzymatic digestion reaction by *Rsa*I enzyme at 37°_{C} for three hours and cutting pattern of genomic segments was observed by electrophoresis on Agarose gel (1.5%).To data analysis of polymorphism results, existence or none existence of each band was recorded as one and zero, respectively. Cluster analysis was conducted using UPGMA and Jacquard coefficients and *NT-SYS V 2.2* program and then its cladogram was drawn.

3. Results

3.1. Morphological traits of rose aphids

Isfahan has its own aphid's fauna for roses like every other place. Wide sampling of all Isfahan places prepared 135 samples totally. According to morphological traits, three aphid species were collected from Isfahan roses. The most abundant species was *Macrosiphum rosae* which? samples were belonged to this species. This species which is known as rose aphid too has been sawn from average to big sizes, with long or spindle shaped bodies in green, yellow, pink and red – brown colors. According to previous reports, this species is distributed all over Iran and can be collected all seasons except summer. The most important morphological characteristics of this species are ?. The second species which was collected less in this study was *Metropolophium dirhodum*. Wingless members of this species are all long spindle – shaped with green color or green – yellow and an obvious bright green back stripe. Antennas are bright and the end of third and fifth part and appendix of final part were dark to black. Antenna length is about 0.6 of total length, and third part has 1 to 3 secondary * in its bottom. The others have a green abdomen without sclerotium spots. Hair formula of tarsus first part was 3-3-3 and tail had 9-12 hairs too. The length of wingless members bodies were from 1.6 to 3.3 mm. This characteristic is completely in accordance to Black man and Eastop (2000) reports. The main host of this insect is rose too and has been reported from many places of Iran. The third species was *aphis gossypii*. This egg shaped insect is about 1.8 mm and is sawn in many colors. Same are green or yellowish green and the other are grey to green. It has been reported from all places of Iran and various hosts. We must announce that some samples were not completely in accordance to identification Key and were ascribed to these species because of showing the most important characteristics. One of important specifics of aphids is similarity of their morphological traits which make their identification difficult [14] .

3.2. RAPD-PCR reaction

In this study 16 aphid samples which had high morphological differences were used to study their genetic diversity. All used primers of this study showed good polymorphism in studied

rose aphids. The size of obtained bands was estimated about 100-2000 bp. Among used primers, UBC 90 and R 108 showed the highest polymorphism with 25 and 24 amplified segments respectively (figure1). According to cluster analysis rose aphids divided into three groups: the first group (A) consisted of 5 aphid samples from various regions of Isfahan. Second group (B) consisted of nine samples which were different in color. Members of this group were black and green and were belonging to *Aphis gossypii* and *Macrosiphum rosae*. Two samples of rose aphids (C2 and V1) were located in C group which C2 was green to grey colored and V7 was brown colored and both were located in *Macrosiphum rosae* species. Results of this study showed that dividing rose aphids by RAPD-PCR has a close relation with their morphological traits but not with their color (figure2).

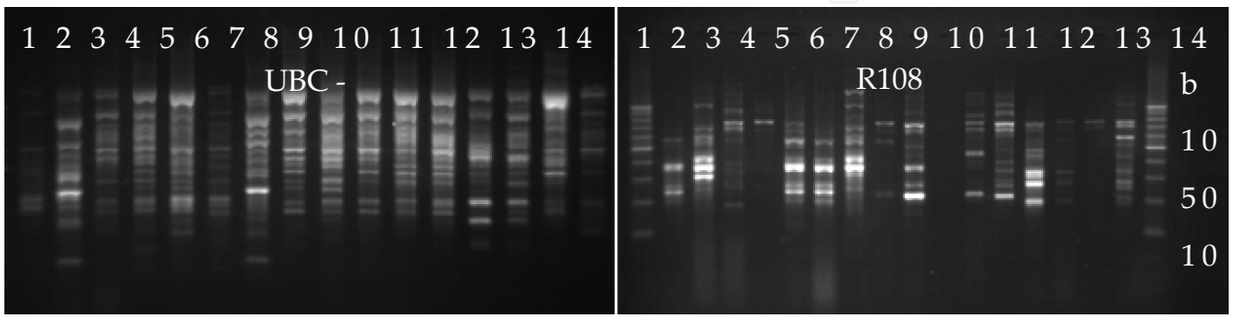

Figure 1. Band figures of rose aphids amplified segments using UBC-90, and R-108 primers in Agarose gel 1.2%

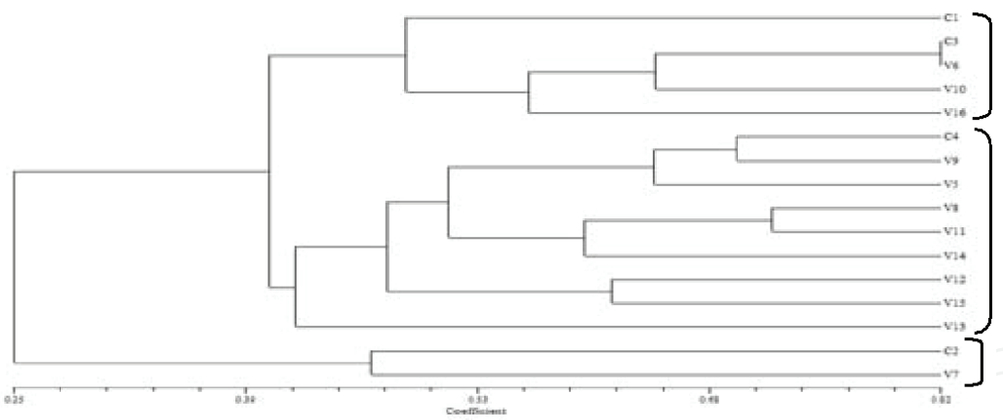

Figure 2. Cluster analysis of rose aphids using data of RAPD-PCR. Group A:, group B:, and group C:

3.3. Genetic diversity of rose aphids using RFLP-PCR

To this, PCR product of mitochondrial gene was digested by *Rsa*I restriction enzyme. The cutting distribution pattern of aphid mitochondrial gene is presented in figure 3. The enzyme could show seven band pattern of polymorphism on mitochondrial genome of aphid samples. Cluster analysis of this polymorphism was done using *NTSYS V 2.2* program (Figure4). As it was shown in cladogram, aphids are dividing in three groups according to RFLP-PCR. Group A were red aphids and were very similar morphologically. In this grouping many of black and green aphids (7 samples) was located in group B and the rest (4 samples) were located in group C. This group-

ing has a high accordance to morphological results of aphid population. For instance, C2, C6, and C9 which were related to numbers 109,105 and 108 (studied samples) respectively were located in one group. In morphological traits all these aphids are red. These results show efficiency of this marker for for classifying and identifying the aphid population, along with their phenotypic characteristics. In second group also samples were located which were very similar morphologically and were different only in color and some other characteristics.

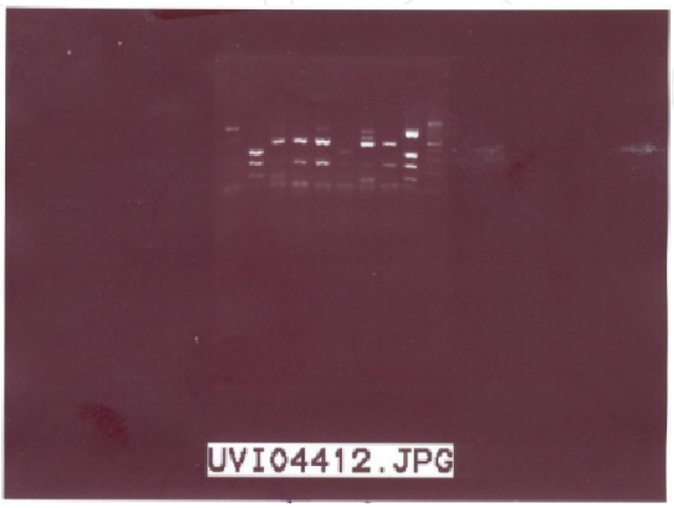

Figure 3. Polymorphism (restriction pattern) of mitochondrial gene of rose aphid in enzyme digesting using RsaI restriction enzyme on Agarose gel 1.5% in TBE buffer

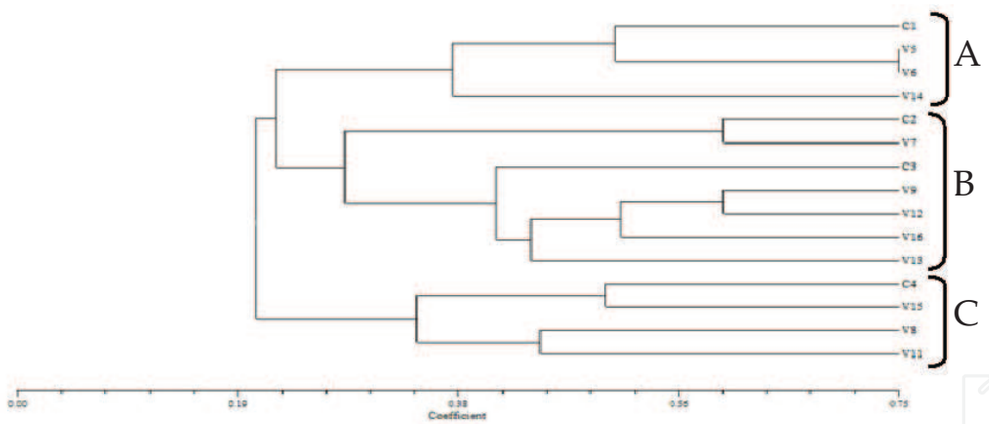

Figure 4. Cladogram of cluster analysis of aphids' polymorphism in RFLP-PCR using NT SYS V2.2 program

4. Summary and conclusion

Population structure of each organism is dependent to amount and distribution of within and between populations genetic diversity. Genetic structure of a population shows evolution history and potential of it for evolution and adaption to environment [5] . Every agricultural ecosystem is facing to environment changes like cultivating resistant cultivars, fungicides, pesticides, fertilizers, irrigation and crop rotation, then pests and plant pathogens are constantly evolving and changing to adapt to these changes. So, having knowledge about power of pest populations in evolution and adaption to environment and host is very important for

completing effective controlling strategies [15] . Then, one of the other goals of studying genetic diversity and population structure is identifying the factor which plays more important role in evolution of that population and also how that factor determines genetic structure of population and its evolution potential[5] . Developing molecular technics and using genetic markers in last decades have led to developing tools and fast, cheap, and accurate methods for identifying creatures. So, polymerase chain reaction (PCR) and molecular methods based on PCR have had an important role in developing biological sciences. In recent years, many efforts have been done for using molecular methods in entomology. For example Wagou et al. (1996) used molecular markers for identifying parasite insects and studying their biology. Genome sequencing and molecular markers is an appropriate replacing method for morphological methods in studying aphids' epidemiology [13] RAPD and AFLP molecular markers have high application as population markers in studies about population diversity of living things especially insects [7] Chen et al (2008) studied genetic diversity of cabbage aphids with molecular methods and reported that when it was not possible to divide aphid populations by morphological traits, RAPD and AFLP markers divided them well. In current study, rose aphids were studied for morphological and molecular properties. According to morphological findings, all collected samples were belonging to three species: *macrosiphum rosae, aphis gossypii,* and *metopolophium dirhodum*. Previous studies confirm it too. These three aphid species have been identified before on roses of Isfahan [16] this study was done to clarify structure of rose aphid's populations from morphological and genetic aspects and also for evaluating use of RAPD and RFLP markers for grouping these populations. UBC-90 primers showed the highest polymorphism in this study. Also, primers could present significant relationship with morphologic traits of rose aphids. Polymorphism analysis of these markers with NTSYS program showed that RFLP markers acted better than RAPD in locating close populations with similar morphological traits in similar clads. Considering high distribution of this pest on various plants, for better realizing the population structure of this pest, it is better to study its genetic diversity on other hosts in various regions. On the whole, results of this study showed that morphological and genetic diversity of this aphid is relatively high, and then effective factors in its creation and expanding must be studied.

5. References

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